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Full Length Research Paper

Ultrasound-assisted degradation of a new bacterial exopolysaccharide WL-26 from *Sphingomonas* sp.

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Ultrasonic degradation of a new exopolysaccharide WL-26 from *Sphingomonas* sp. was made over the frequency range 200 to 1200 Hz and polymer concentrations of 3, 5, 10 and 20 g/L using high performance anion exchange pulsed-amperometric detection chromatography (HPAEC–PAD) and infrared spectroscopy. Sonication was more efficient with less concentrated polysaccharide solutions, high ultrasonic frequency, long duration of ultrasonic irradiation and degradation continued until a limiting molecular weight was attained. Results show that HPAEC–PAD revealed WL-26 to be an acidic polysaccharide composed of rhamnose, glucose, mannose, galactose and glucuronic acid in the molar ratio of 10:9:3:1:3 distinctly different from welan gum which does not contain galactose.

Key words: Exopolysaccharide WL-26, ultrasonic degradation, infrared spectroscopy.

INTRODUCTION

Microbial exopolysaccharides (EPS) have been used in many industrial applications as thickeners in food, cosmetics and pharmaceuticals. Xanthan gum is perhaps one of the most frequently used biogums as a stabilizer, emulsifier and rheological control agent (Balsara et al., 1992). In nature, microbial polysaccharides often occur as capsules around bacterial cells to prevent dehydration and for attachment to solid surfaces. Microbial EPS has been studied extensively as a result of its versatile structure, properties and functionalities.

Microbial EPS produced *by Sphingomonas* species are referred to as sphingans and include well-known members such as gellan and welan gums (Lobas et al., 1992; Martins et al., 1996; Hashimoto et al., 1998; Fialho et al., 1999). These sphingans have similar backbone structures and include gellan (Kang and Veeder, 1982), S-88 (Kang and Veeder, 1985), welan (Kwon et al., 1987) and rhamsan (Podolsak et al., 1996), all of which has a linear repeating tetrasaccharide containing D-glucose, D-glucuronic acid and L-rhamnose, (Pollock, 2002). Gellan is an exceedingly versatile gelling agent used by the food industry. Its unique functionality at elevated temperature makes gellan especially useful as a gelling agent in media for the growth of microorganisms particularly applicable as a gelling agent at high temperature when other gelling agents, such as agar, does not function (Lin and Casida, 1984). Welan solution is stable and functional at relatively high temperature and thus very useful for oil field applications (Kang et al., 1983).

Due to the high viscosity of some polysaccharides, analytical methodologies such as one-dimensional-nuclear magnetic resonance (1D-NMR), 2D-NMR and mass spectrometry are not always efficient to elucidate their chemical structure. The polysaccharide must first be depolymerized using various methods including exposure to heat and light, and treatment with chemical reagents and enzymes (Madras and Chattopadhyay, 2001).

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Abbreviation: HPAEC–PAD, High performance anion exchange pulsed-amperometric detection chromatography.

However, such treatments may lead to non-specific cleavage of glycosidic bonds and chemical changes, thereby preventing accurate structural determinations (Voragen et al., 1993).

The application of ultrasound for the purpose of polymer degradation can be traced back to the 1930s (Price et al., 1994). The technique was subsequently proved to be a highly advantageous method for deploymerizing macromolecules because it reduces the molecular weight simply by splitting the most susceptible chemical bond without causing any changes in the chemical nature of the polymer. Ultrasound has been used for the degradation of a wide range of polymers (Price, 1990). Ultrasonic power and ultrasonic time are the main factors to influence this treatment (Shen et al., 2006; Zheng et al., 2008). The ultrasonic degradation process has several unique features such as its non-random nature and molecular weight dependence that distinguish it from thermal or photochemical degradation (Price and Smith, 1991). The scission of polymer chains in a solution occurs preferentially near the middle of the chain (Price and Smith, 1993a; Price and Smith, 1993b; Gronroos et al., 2001).

The new exopolysaccharide WL-26, produced by *Sphingomonas* sp. ATCC 31555, exhibits a high viscosity even at low concentration. Furthermore, WL-26 is stable at high ionic concentrations and over a wide range of pH and temperature. The objective of this study was to determine the optimum conditions for ultrasonic depolymerization of WL-26 in order to degrade the polysaccharide into fragments with lower molecular weight and viscosity and facilitate its structural characterization.

MATERIALS AND METHODS

Strain and fermentation conditions

Sphingomonas sp. ATCC 31555 (formerly known as *Alcaligenes* sp. at the time of its isolation) was used in this work. It was cultivated on a defined medium containing 40 gL^{-1} sucrose, 4 gL^{-1} complex nitrogen (20% sodium nitrate and 80% yeast powder), 2 gL^{-1} KH_2PO_4, 0.1 gL^{-1} $MgSO_4$ and 0.5 mLL^{-1} $FeSO_4$. Exponentially growing seed cultures for submerged fermentations were used for inoculation at a volume ratio of 1:10. A 3-L Biostat C plus stainless steel reactor from B. Braun (Sartorius Stedim Biotech AG) initially with lower nutrient followed by feeding the broth with higher nutrient culture several times according to the pH of the broth was used for the fermentation. Under this condition the difficulties of broth aeration can be alleviated with higher productivity of WL-26. The other fermentation parameters were: operating volume, 2 L; temperature, 30°C; stirrer speed, 400 rpm; aeration, 2 L/min. The pH-value was set initially at 7.0. The dissolved oxygen was kept above 20% by changing aeration rate in various phases during the fermentation process.

Chemicals and reagents

Dextrans T-140, 67, 41, 27, 15, 8, 5, 2.5, 1.2, and the monosaccharide standards, D-Glc, D-Man, D-Gal, L-Fuc, L-Rha, D-Ara, D-Xyl, GalA, GluA were purchased from Sigma–Aldrich. All reagents were of analytical grade.

Extraction and purification of WL-26

The culture broth was precipitated with ethanol (95%, v/v) until the final alcohol concentration reached 75%. The precipitated crude materials was further washed with 75% (v/v) ethanol and resuspended in distilled water to the original volume, precipitated, centrifuged and lyophilized. A portion of lyophilized polysaccharide (0.2 g) was dissolved in 2000 ml water, centrifuged at 12,000 rpm (26,000 × g) at room temperature for 20 min. The supernatant was concentrated into one-tenth of the original volume, dialyzed in a dialysis bag with a permeability size of 0.8 to 10 kDa in distilled water for four days, evaporated at reduced pressure at 40°C and lyophilized. The resulting product is designated as crude polysaccharide WL-26.

Determination of molecular weight of WL-26

Homogeneity and molecular weight determinations were carried out by high performance liquid chromatography (HPLC) linked to a gel-filtration column TSK PWXL 6000. Samples (10 µL 0.2% (w/v) solution in 0.1 M phosphate buffered saline (PBS buffer)) were applied to the column and eluted at 35 ± 0.1°C with 0.1 M PBS and 0.3 M $NaNO_3$ (pH 7.0) at a flow rate of 0.5 mL/min. The column was calibrated using dextrans T-140, 67, 41, 27, 15, 8, 5, 2.5 and 1.2.

Rheological properties of WL-26 solutions

WL-26 solutions (3, 5, 10 and 20 g/L) were prepared gravimetrically using an analytical balance (Sartorius BS 210 S) with a precision of ± 1×10^{-4} g. The relative viscosity (η_r) values of the WL-26 solutions as a function of shear rate and concentration were determined over shear rates ranging from 0.3 to 100 rpm at 25°C using Brookfield DV-I^+ (Brookfield Engineering Labs., Inc.，U.S.A) equipped with a S64 spindle.

Monosaccharide analysis

Samples (2 mg) were hydrolyzed with 2 M trifluoroacetic acid (TFA) at 110°C for 4 h, and the resulting monosaccharides were identified by high-performance anion exchange pulsed-amperometric detection chromatography (HPAEC–PAD). Monosaccharide components and percentage composition were determined using D-Gal, D-Glc, D-Ara, L-Fuc, L-Rha, D-Man, D-Xyl, GluA, and GalA standards.

Ultrasonic degradation

Solutions (50 mL) of WL-26 in distilled water (3, 5, 10 and 20 g L^{-1}) were placed in plastic tubes (100 mL) and subjected to ultrasound treatment over the frequency range 400 to 1200 Hz using a 400 JY 99-II ultrasonic generator (Ningbo Scientz Biotechnology Co., Ltd, Ningbo, China.) at 25°C. The probe was immersed 1 cm below the liquid surface each time. Samples were removed from the tube periodically and viscosity values were measured at 25°C using a Brookfield DV-I^+ viscometer (Brookfield Engineering Labs., Inc., U.S.A).

Infrared spectroscopy

Samples were incorporated into KBr discs and analyzed using a

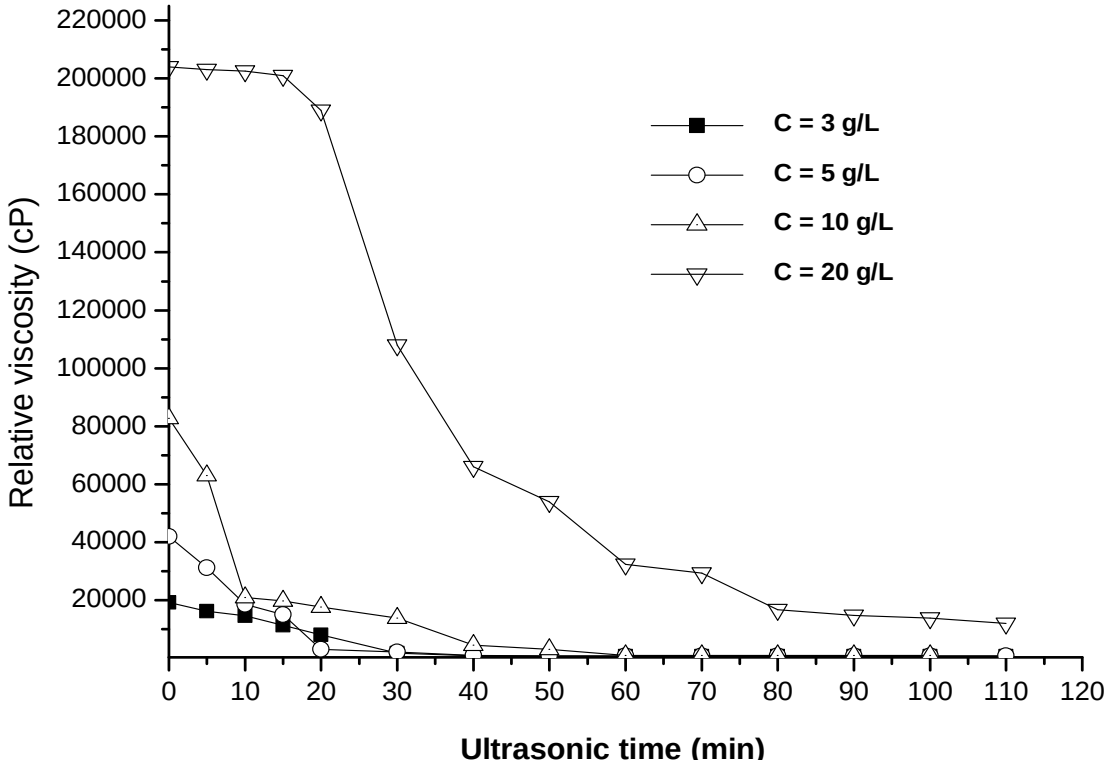


Figure 1. The relationship between relative viscosity (η_r) and sonication time for different concentration of WL-26 aqueous solutions.

599B Fourier transform infrared (FT-IR) spectrophotometer (Perkin-Elmer, Waltham, USA).

RESULTS

Effect of WL-26 concentration on depolymerization

Sonication was carried out for four different WL-26 concentrations and the frequency of the ultrasound was set at 600 Hz. As shown in Figure 1, it is apparent that the viscosity decreases with sonication time and tends to have a constant value. When the WL-26 concentration increased, a longer sonication time was required. The viscosity was lower in 40 min in the 3 g/L WL-26 solutions. These results indicate that the extent of degradation is more pronounced in the more dilute solutions. The reason might be that the probability of chemical bond scission caused by efficient shearing in the polymer chain is greater in dilute solution (Gronroos et al., 2001; Taghizadeh and Bahadori, 2009).

Effect of sonication frequency on the degradation rate

In order to test the effect on sonication frequency on the depolarization of WL-26, WL-26 solutions (5 g/L) were treated at five different ultrasonic frequencies (400, 600, 800, 1000, and 1200 Hz) at 25°C. As shown in Figure 2, when the higher ultrasound frequency increased, shorter sonication time is required to reduce the viscosity. The viscosity of polymer solution decreased significantly over 90% at 1200 Hz within 20 min.

Effect of ultrasonic time on the molecular weight of WL-26

The molecular weight is important parameter to evaluate the depolymerization effect of the sonication treatment. As shown in Figure 3, the reduction of molecular weight with sonication time tends to have a constant value. A marked reduction of the M_w of polymer was observed during the initial 90 min when the molecular weight decreased by 68%. After further ultrasonication, the reduction of molecular weight became slower, and during the following 150 min it decreased only by 16%. The degradation of molecules continued only to a certain limiting molecular weight. The results are similar to other previous studies on the sonication degradation of various different polysaccharides (Mason and Peters, 2002; Machova et al., 1999).

This study confirms the general assumption that the shear forces generated by the rapid motion of the solvent

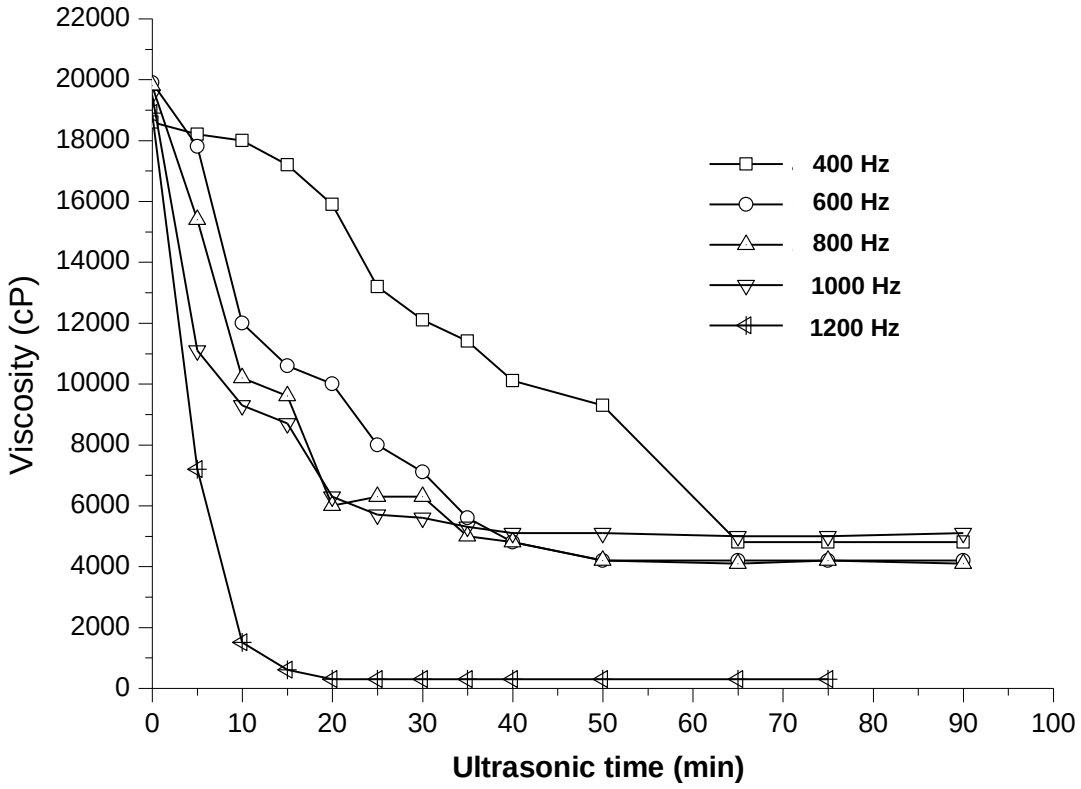
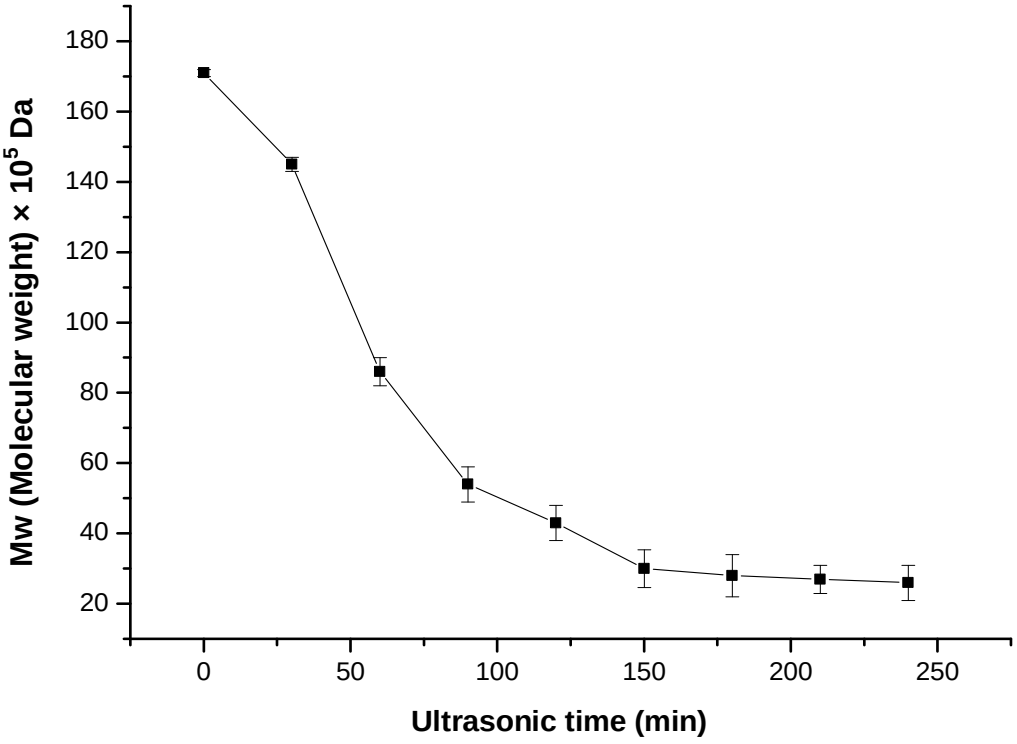


Figure 2. The relationship between relative viscosity (η_r) and sonication time at different ultrasonic frequency.



Figuer 3. Variation of molecular weight at different sonication time (WL-26 of 5 g/L).

Table 1. Sugar composition of sonication degradation WI-26 expressed as mol%.

Sonication time (h)	Sugar composition				
	Glc	Rha	Man	Gal	GlcA
0	9.11 ± 0.2133	9.98 ± 0.6721	3.35 ± 0.4412	0.99 ± 0.2534	3.33 ± 0.3212
0.5	8.79 ± 0.1988	9.12 ± 0.3112	3.18 ± 0.4312	1.02 ± 0.3232	4.23 ± 0.3412
1.0	8.37 ± 0.2311	9.21 ± 0.3724	2.98 ± 0.3314	1.23 ± 0.2532	4.88 ± 0.1768
1.5	7.32 ± 0.1456	8.19 ± 0.3124	3.24 ± 0.4523	0.93 ± 0.1632	5.00 ± 0.1142
2.0	8.10 ± 0.2312	8.95 ± 0.6316	2.76 ± 0.2112	0.89 ± 0.1321	4.94 ± 0.4211
2.5	7.05 ± 0.4123	7.01 ± 0.4123	2.44 ± 0.5234	1.11 ± 0.2112	4.41 ± 0.4333
3.0	8.41 ± 0.1353	9.31 ± 0.0134	3.44 ± 0.1022	1.11 ± 0.0988	3.78 ± 0.2412
3.5	7.28 ± 0.2563	8.41 ± 0.5213	3.09 ± 0.1123	1.23 ± 0.1789	5.02 ± 0.3111
4.0	8.14 ± 0.1876	8.34 ± 0.2453	3.09 ± 0.2241	0.96 ± 0.2134	4.13 ± 0.0989

Experiments were performed three times with excellent reproducibility. Rha, Rhamnose; Glc, glucose; Man, mannose; Gal, galactose; GlcA, glucuronic acid.

following cavitational collapse are responsible for the breakage of the chemical bonds within the polymer. The effect of polymer concentration can be interpreted in terms of the increase in viscosity with concentration, causing the molecules to become less mobile in solution and the velocity gradients around the collapsing bubbles become smaller. In addition, this observation strengthens the claim that ultrasonic degradation, unlike chemical or thermal decomposition, is a nonrandom process with cleavage taking place presumably at the center of the molecule.

The monosaccharide from the WL-26 sonication mixtures were analyzed by anion-exchange chromatography. As shown in Table 1, it is apparent that sonication time only changes slightly the molarity of glucose, rhamnose, and glucuronic acid, and did not affect the molarity of mannose and galactose relative to the sonication time.

Structural identification by infrared spectroscopy

To identify the structural difference of the treated WL-26 by sonication, infrared spectroscopy was performed as earlier described. As shown in Figure 4, sonication treated samples had absorbance bands at 3200 to 3400, 2933 to 2981, 1725 to 1730, 1200 to1000, and 1015 to 1060 cm^{-1}, respectively (Figures 4A, B and C). Absorbance bands at 3200 to 3400 cm^{-1} indicates that it has OH stretch, intermolecular H-bridge between the OH groups assignment; 2933 to 2981 cm^{-1} means CH_2 and symmetric stretch assignment; 1725 to 1730 cm^{-1} means C=O stretch from acetyl- or COOH groups assignment while 1200 to 1000 cm^{-1} have three absorption peaks means they all have pyranose. However, as compared to the initial sample, there is no obviously change. The results indicate that sonication does not degrade the polysaccharide chain structure, but only disrupt the sugar chemical bonds in the center of the chains.

DISCUSSION

Information about the structure of polysaccharides can be obtained by both chemical and sonication degradation of the polysaccharide fractions, followed by the identification of the resulting degradation products. However, chemical hydrolysis with dilute acid cleaves the glycosidic bonds in a rather unspecific way, which prevents the conversion of the experimental data into a hypothetical structure of the biopolymer. Chemical treatment, such as acid hydrolysis, is often time and energy consuming, involves use of chemicals and generates waste. Moreover, being a multi-parameter process, it is not always easy to control, although considerable progress in this technique has been achieved (Wojtasz-Pajak et al., 1998). Ultrasonication has been used to depolymerize various biopolymers including DNA, dextran and bacterial capsular polysaccharide without alteration of their chemical structure (Szu et al., 1986). Sonication has high substrate specificity and produces characteristic polymers. Consequently, it is a valuable tool in structure elucidation. It has been demonstrated on chitosan and starch that treatment with 360 kHz ultrasound in aqueous solution was an efficient procedure for reduction of molecular weight of the polysaccharides. Under these conditions, degradation was caused by OH radicals and mechanochemical effects (Renata et al., 2005). Ultrasonication of the polysaccharide schizophyllan produced by *Schizophyllum commune*, performed in dilute aqueous solution, resulted in the formation of a lower-molecular-weight polysaccharide with conserved primary structure and antitumor activity (Yanaki et al., 1983).

Welan gum is an extracellular polysaccharide produced by the bacterium *Sphingomonas* ATCC 31555 (the same microorganism that produced WL-26). It was initially

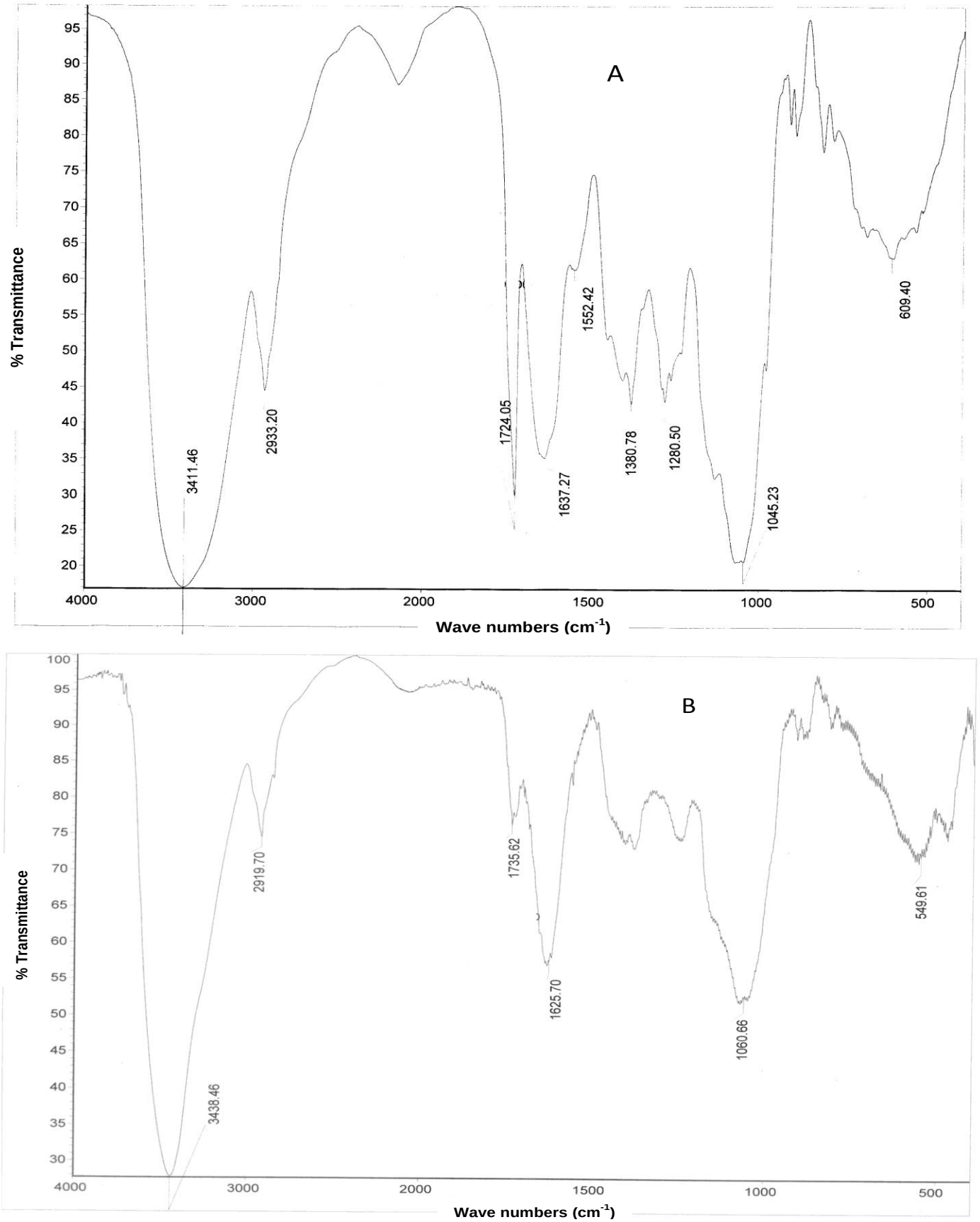


Figure 4. IR spectrum of WL-26 from different sonication treatment time (A, 0 h; B, 0.5 h; C, 4 h).

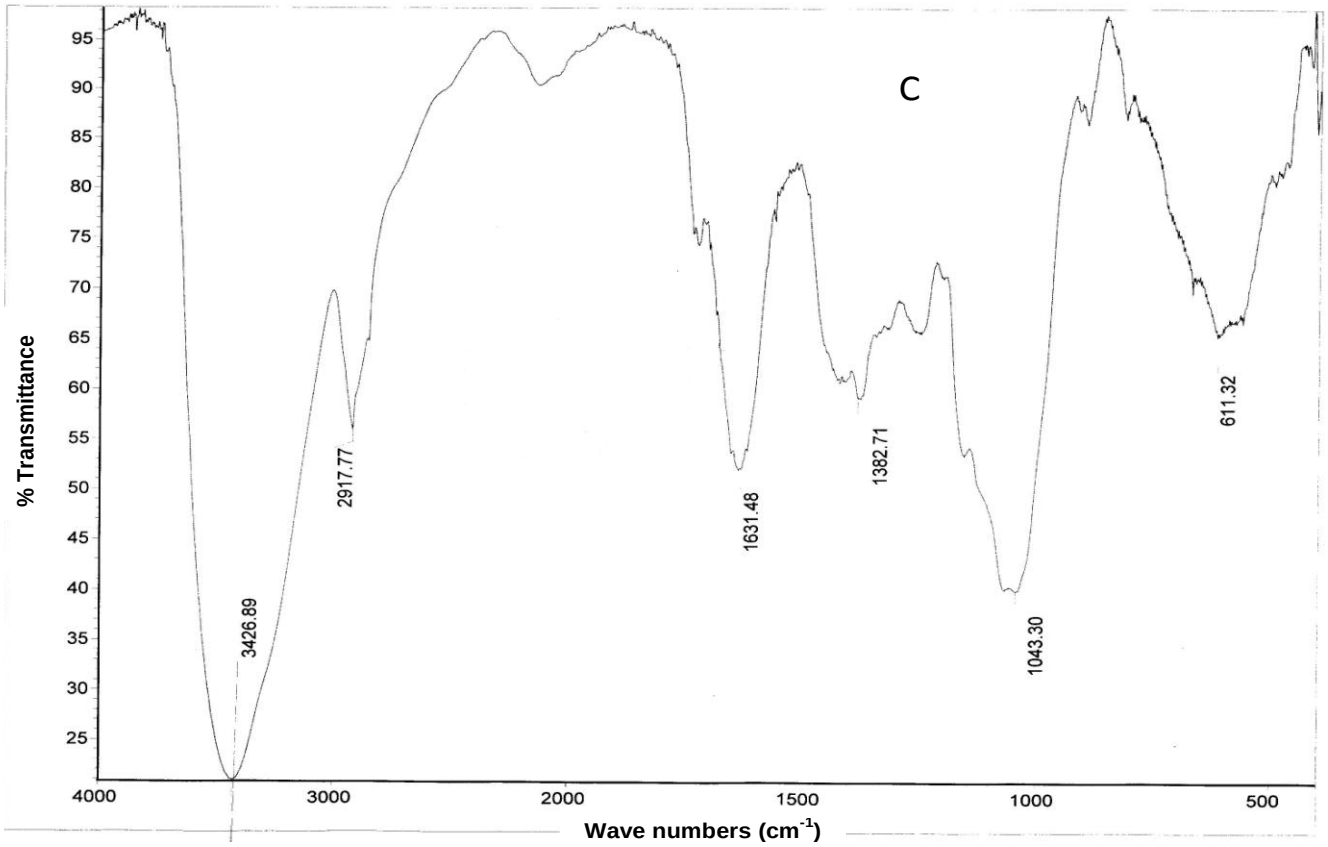


Figure 4. Contd.

known as S-130 and has been commercialized by the Kelco Division of Merck and Company under the trade name Biozan in the 1980s. Aqueous solutions of welan gum exhibit a high viscosity at low shear rate which is maintained at high temperatures. It is remarkably insensitive to pH and to the concentration of calcium and other ions. The chemical structure of welan comprises a backbone with the tetrasaccharide repeating unit ←3)-β-D-Glcp-(1 ←4)- β-D-GluA-(1← 4)- β-D-Glcp-(1←4)-L-Rhap-(1← and a side chain of either -L-Rhap or ~-L-Manp joined (1→ 3) to the 4-1 linked β-D-Glcp and approximately two-thirds of side chain units are ~-L-Rhap and these are randomly distributed (Jansson et al., 1985). In native welan, approximately 85% of the 3-1inked β-D-Glcp have O-acetyl groups in the 2-position (Phillip et al., 1986). They all have high viscosities at low-shear rates and exhibit shear-thinning behaviors. However, polysaccharide WL-26 has a distinctly different chemical structure comparing to welan gum. WL-26 contains galactose which was not part of the chemical composition of welan gum. At present, it is not known why this same *Sphingomonas* sp. ATCC 31555 is capable of producing both welan gum and WL-26 polysaccharide. This report detailed a preliminary study on the structure of WL-26. Further research will focus on the elucidation of the structure of the polysaccharides with different molecular weight formed after degradation of WL-26 fractionated with ultrasonic and partial acid hydrolysis as well as chromatography techniques, GC-MS and NMR analyses.

ACKNOWLEDGEMENT

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